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Annual Report, January 1 - December 31, 1952

Contract No. Nonr - 146 (00) University of Texas

Title of Project: Spore Formation and Spore Germination in Bacteria

Responsible Investigator: J.W. Foster, Professor of Bacteriology

Objectives: Study of the fundamental biological and biochemical nature of those processes.

#### Part A Spore Formation

Our work up to the present has permitted the formulation of a new concept of sporogenesis in aerobic bacteria. Discovery that washed vegetative cells of various species of Bacillus sporulate according to a predictable pattern provided an experimental approach to basic aspects uncomplicated by growth which otherwise takes place when exogenous nutrients are present. Key experimentally obtained points were: No sporulation takes place for about 8-10 hours during shaking in distilled water in the absence of nutrients, then a triggerlike effect is observed, 70-90 per cent of all the cells sporulating suddenly and within a 2 hour period. These spores are indistinguishable from those produced in complete growth media, as judged by heat resistance. This experiment demonstrates that sporogenesis can be a strictly endogenous process. As a means of distinguishing this event from sporogenesis occurring in growth media, and to describe the situation where the vegetative cell, in the complete absence of exogenous nutrition furnished all the requirements for spore formation we propose the term "endotrophic sporulation".

A great many factors influencing endotrophic sporulation have been studied. Low concentrations of readily assimilable carbon sources, in particular carbohydrate, have a unique suppressive effect on endotrophic sporulation. This suppression is observed only when the carbohydrate (glucose) is added at any time up to about 5 hours to the cell suspension shaking in distilled water. Added after the 5th hour to 8-10 hours, sporogenesis is not suppressed, though no spores have been formed at the time of addition. A change in the vegetative cell taking place well before visible sporogenesis committing the cell to sporulation evidently takes place during the first 5 hours, so that the sporulation process is not thereafter reversible by the addition of glucose. We interpret this change as some kind of endogenous synthesis, and that added prior to 5 hours availability of carbon in the form of glucose induces a competitive synthesis. The latter preempts sporogenesis by using up intracellular precursors essential for the spore formation. These were postulated to be low molecular weight nitrogenous compounds. Confirmation of this idea was obtained in an experiment in which the presence of a small amount of ammonia was able to restore sporogenesis in the presence of glucose. Involvement of amino acids and purines in endotrophic sporulation was established by showing that certain amino acid and purine analogue inhibitors do inhibit sporulation and this inhibition is completely reversed the presence of the corresponding metabolite.

The above demonstrate that small molecular weight substances are essential for endotrophic sporogenesis and implies that to some extent the spore formation involves a biosynthesis using small molecular weight metabolites. This de novo endogenous synthesis of spore material is a new concept of sporogenesis and must be evaluated along with the classical concept of an agglomeration or concentration of cytoplasm or cytoplasm granules as responsible for the appearance of the spore body.

The synthesis occurring between the 5th and 8th hours is not made from the amount of free small molecular weight substances normally existing in the vegetative cell because their concentrations are too small. Our idea is that they are furnished in a low steady state concentration from a bound-form pool, the latter being probably enzymatic proteins characteristic of the vegetative cell. The latter would therefore be used up as sporogenesis proceeds, in a manner analogous to the disappearance of the enzyme protein pool during synthesis of adaptive enzymes (Spiegelman). Since both sporogenesis and adaptive enzyme synthesis require the non-specific protein pool in the vegetative cell these processes should be competitive and mutually exclusive in any one cell. Vegetative cells of *B. mycoides* between 1-5 hours adaptively attack maltose, but not at 8 hours, when the irreversible biosynthesis preceding sporulation has used up the precursor pool, thus precluding adaptive enzyme synthesis, which is dependent on that pool. Also vegetative cells obtained protein-poor by growth in low nitrogen containing media, do not display endotrophic sporulation, supporting the hypothesis that a protein pool is essential to furnish the building blocks for spore synthesis.

According to the above sporulation will be accompanied by a loss of the enzymatic proteins characteristic of vegetative cells. Seventeen enzyme systems have been studied in this connection. Under conditions in which extracts of vegetative cells display easily measurable activity for 17 enzymes, extracts of spores displayed no detectable activity. Thus the enzymes of the vegetative cells do indeed disappear, a finding consistent with the above hypothesis, but by no means establishing it.

Degradation of the vegetative proteins has been studied with  $S^{35}$ -labeled cells. Criterion of the degradation was the appearance of soluble radioactivity over hourly intervals during the course of endotrophic sporulation. To trap the intermediate labeled methionine a pool of unlabeled methionine was present. Release of soluble radioactivity reached a peak between the 4th and 5th hour, and dropped off sharply thereafter. This fits the hypothesis: degradation rises to a peak early. The accumulation of soluble radioactivity naturally would be less during the 5-10 hours when active resynthesis using the soluble building blocks occurs.

Test of a period of active synthesis during the "glucose-irreversible" phase namely 5-8 hours, was also made possible by means of  $S^{35}$ -methionine. The hourly rate of protein synthesis was studied, using as the criterion incorporation of  $S^{35}$ -methionine into insoluble protein form. Matching the hypothesis, protein synthesis reached a sharp peak between the 5th and 8th hours.

These experiments strongly support the hypothesis of spore synthesis at the expense of vegetative enzyme protein degradation, and that there is a time sequence dependence.

More information relative to amino acid and purine intermediacy in endotrophic sporulation has been obtained by quantitative analysis of certain amino acids during amino acid analogue inhibition. Of special significance is the finding that any single analogue which inhibits utilization of the corresponding amino acid, prevents utilization non-specifically of other amino acids. Thus, utilization of any one amino acid necessitates concomitant utilization of all, this find is in complete agreement with the idea of protein synthesis, which would require all or none of the amino acids if it is to occur.

In these experiments the intracellular content of certain amino acids diminishes distinctly as the cell metamorphoses to a spore. In those cells inhibited by an analogue, the content of amino acids does not diminish or does so much less than in uninhibited cells. Direct tests of aspects of amino acids during sporogenesis and especially inhibition of this uptake by analogues yielded conclusive results when incorporation of  $S^{35}$ -methionine into insoluble protein was employed as the criterion. Similarly, endotrophic sporulation is known to be extremely sensitive to acidity, so that at pH 5.0 - 5.5 the process is inhibited.  $S^{35}$ -methionine uptake is also halted, indicating that pH inhibition is concerned with the biosynthetic steps in sporulation. Also consonant with all the information and theory relative to glucose action, are experiments which show that  $S^{35}$ -methionine uptake is not depressed while sporogenesis is inhibited by glucose, but is accentuated.

As a corollary of the enzymatic analyses described earlier, assay of certain "B" vitamins has been made during the course of endotrophic sporulation. Each of the 5 vitamins studied was present in spores at a substantially lower level than in the vegetative cells whence the spores were derived, the evidence in some vitamins indicates they are destroyed; in others they are released into the suspending water.

### Part B Spore Germination

The approach here has been predicated on the idea that the germination process in its biochemical essence is the reverse of the sporulation process as described in Part A. Our criterion of germination is the acquisition of heat lability. This transpires before visible signs of vegetation. Our concept is that the labilization occurring during germination is the consequence enzyme of ~~synthesis, this taking~~ place at the expense of the material characteristic of the spore. The latter would represent a "pool" whence come the materials essential for enzyme synthesis. The conversion of heat-stable precursor pool to the heat-labile enzymes characteristic of vegetative cytoplasm would account for the loss of heat resistance of the spore during germination.

Extracts of spores allowed to just become heat labile exhibited small but definite enzyme activity with respect to 17 different enzymes tested. Thus an initial synthesis of vegetative enzymes had occurred prior to any visible evidence of growth. Thus active synthesis of proteins, presumably enzymatic proteins, takes place during the first 50 minutes incubation in yeast extract-glucose broth, in which time no morphological change occurs. More direct evidence for synthetic activity during this early phase was obtained from an experiment in which  $S^{35}$ -methionine was taken up from the medium and converted into insoluble (protein) form. Evidence of degradation of spore protein to low molecular weight compounds as a prelude to subsequent enzyme synthesis comes from a study of release in soluble form of radioactivity during the heat labilization phase of  $S^{35}$ labeled spores.